



# Soluble form of Fas and Fas ligand in serum and bronchoalveolar lavage fluid of individuals infected with human T-lymphotropic virus type 1

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Received 7 July 2003; accepted 25 September 2003

## KEYWORDS

Fas;  
Fas ligand;  
HTLV-1;  
Bronchoalveolar lavage fluid

**Summary** Human T-lymphotropic virus type 1 (HTLV-1) carriers are known to develop pulmonary complications characterized by T-lymphocytic alveolitis. The aim of this study was to determine the profile and role of soluble Fas (sFas) and sFas ligand (sFasL) in the lung of asymptomatic HTLV-1 carriers. We measured sFas and sFasL levels in serum and bronchoalveolar lavage fluid (BALF) of 16 seropositive asymptomatic HTLV-I carriers and 32 healthy subjects. The serum levels of both sFas and sFasL were significantly higher in HTLV-I carriers than in the control. In BALF, the percentage of lymphocytes and CD4 positive T-cells, and the levels of sFasL were also significantly higher in asymptomatic carriers than the control, but there were no significant differences in sFas levels between the two groups. There was a significant correlation between BALF sFasL levels and serum sFasL levels and percentage of CD4 positive T-cells in BALF. Our results suggest that the increased levels of sFasL in the lung of asymptomatic HTLV-I carriers are associated with accumulation of CD4 positive T-cells, and that resistance to apoptosis in HTLV-1 infected T-cells and overproduction of sFasL could contribute to T-lymphocytic alveolitis by down-regulating Fas–FasL mediated apoptosis.

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## Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that causes adult T-cell leukemia (ATL)<sup>1,2</sup>

**Abbreviations:** ATL, adult T-cell leukemia; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; HAM/TSP, HTLV-1 associated myelopathy/tropical spastic paraparesis; HAU, HTLV-1 uveitis; HTLV-1, human T-lymphotropic virus type 1; sFas, soluble Fas; sFasL, soluble Fas ligand; sIL-2R, soluble interleukin-2 receptor

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and is also associated with several non-malignant disorders such as HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP),<sup>3,4</sup> HTLV-1 uveitis (HAU),<sup>5</sup> and HTLV-1 associated arthropathy.<sup>6</sup> With regard to pulmonary involvement in HTLV-1 infection, patients with HAM/TSP and HAU frequently exhibit pulmonary complications characterized by T-lymphocytic alveolitis.<sup>7,8</sup> Maruyama and coworkers<sup>9</sup> also reported a strong association between bronchoalveolar disorders and patients with HTLV-1 infection, even healthy carriers of HTLV-1, and have proposed the term HTLV-1-associated bronchopneumopathy for these pathogenic conditions.

Fas, a member of the tumor necrosis factor (TNF) receptor family<sup>10</sup>, was originally described as a cell surface membrane protein (mFas), but a soluble Fas (sFas) in serum that lacks the transmembrane domain due to alternative splicing has been isolated.<sup>11</sup> sFas blocks apoptosis by inhibiting the binding of Fas ligand (FasL) to Fas on the cell membrane.<sup>12</sup> FasL, a 40-kDa type membrane protein belonging to the TNF family,<sup>13</sup> induces apoptosis by binding to Fas receptor. FasL is predominantly expressed on the cell surface membrane (mFasL) in activated T-cells and natural killer cells,<sup>14</sup> whereas Fas is expressed in various cells and tissues. FasL is converted to a soluble form—soluble Fas ligand (sFasL)—by the action of a matrix metalloproteinase-like enzyme.<sup>15</sup> The recent studies have revealed that the human sFasL did not kill Fas-expressing leukemic T-cell line Jurkat, lymphoma cells and primary mouse hepatocytes.<sup>15–17</sup> Soluble murine FasL also loses the ability to induce apoptosis and inhibits membrane FasL-induced cytotoxicity.<sup>18</sup> Furthermore, high levels of sFasL in the serum of the cancer patients are associated with poor prognosis.<sup>19–21</sup> These findings suggest that sFasL could block cell apoptosis by the immune system. In patients infected with HTLV-1, serum and cerebrospinal fluid (CSF) levels of sFas and sFasL have already been reported. In these studies, high levels of sFas were found in sera of HAM/TSP patients<sup>22,23</sup> and high levels of sFasL were also noted in sera and CSF samples from HAM/TSP and ATL patients.<sup>24</sup> These studies suggest that sFasL may contribute to the inflammatory process and subsequent tissue damage in patients infected with HTLV-I.

The present study was designed to clarify the involvement of sFas and sFasL in lung inflammation of HTLV-1 carriers. For this purpose, we compared the levels of sFas and sFasL in serum and bronchoalveolar lavage fluid (BALF) of asymptomatic HTLV-1 carriers and healthy subjects.

## Materials and methods

### Study population

The study population consisted of 16 seropositive asymptomatic HTLV-1 carriers (10 males and six females, aged  $54 \pm 11$  years, mean  $\pm$  SD) who were referred to our hospital and performed bronchoalveolar lavage between September 1992 and December 1998, and 32 healthy subjects (25 males and seven females, aged  $24 \pm 4$  years). Seven HTLV-

1 carriers and three controls were smokers. Seropositivity was determined using both the particle agglutination and indirect immunofluorescence tests. All HTLV-I carriers had no other viral infections, no other HTLV-1 associated diseases and normal chest radiographic findings. All healthy subjects were HTLV-1 seronegative, also free of any viral infection and normal chest radiographic findings. Informed consent was obtained from all carriers and healthy subjects prior to the commencement of the study, and the human experimentation guidelines of Nagasaki University were followed in conducting clinical research.

### Bronchoalveolar lavage and cell preparations

Bronchoalveolar lavage was performed as described previously.<sup>25</sup> The patient was premedicated intramuscularly with atropine (0.5 mg). After local anesthesia with 4% lidocaine, a flexible bronchoscope (BF P200, Olympus, Tokyo) was wedged into a subsegment of the right middle lobe for lavage. A 50-ml sterile physiological saline solution at body temperature was instilled through the bronchoscope and the fluid was immediately retrieved by gentle suction using a sterile syringe. Saline instillation was performed four times. The collected lavage fluid was passed through two sheets of gauze and centrifuged at  $400 \times g$  for 10 min at  $4^\circ\text{C}$  and the supernatant was stored at  $-80^\circ\text{C}$  until use. After washing twice with phosphate buffered saline solution, cells were suspended with 10% heat inactivated fetal calf serum (FCS) and counted using a hemocytometer. An aliquot was then adjusted to  $2 \times 10^5$  cells/ml and 0.2 ml sample of each cell suspension was spun down onto a glass slide at  $160 \times g$  for 2 min using a cytocentrifuge (cytospin 2, Shandon Instruments, PA). The slides were dried, fixed, and then stained using the May–Giemsa method. Two hundred cells were identified under the photomicroscope. The remaining cells were resuspended in RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with 10% FCS, and incubated in plastic dishes for 60 min at  $37^\circ\text{C}$  in humidified 5%  $\text{CO}_2$ –air environment. More than 90% of non-adherent cells collected for flow cytometric analysis were viable as determined by the trypan blue exclusion test.

### Monoclonal antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (interleukin-2 receptor (IL-2R)- $\alpha$ ) antibody and phycoerythrin (PE)-conjugated anti-CD3, CD4,

CD8 antibodies were used in the study. Mouse IgG1 and IgG2a conjugated with FITC or PE (Becton Dickinson, San Jose, CA) were used to determine the borderline between stained and unstained cells in flow cytometric analysis.

### Measurement of sFas and sFasL by ELISA

Serum and BALF concentrations of sFas and sFasL were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (sFas, sFasL ELISA kit, MBL, Nagoya, Japan). Briefly, samples to be measured or standards were incubated in wells coated with anti-Fas polyclonal antibody or anti-FasL monoclonal antibody. After washing, a peroxidase-conjugated anti-Fas monoclonal antibody or anti-FasL monoclonal antibody was added to the well. After incubation the wells were washed, then the peroxidase substrate was mixed with the chromogen and allowed to incubate for an additional period of time. An acid solution was then added to each well to terminate the enzymatic reaction and stabilize the developed color. The optical density of each well was measured at 450 nm using a microplate reader. The concentrations of sFas and sFasL were determined from the respective calibration curves constructed using reference standards. Lower limits of detection were 8 pg/ml for sFas and 6 pg/ml for sFasL.

### Statistical analysis

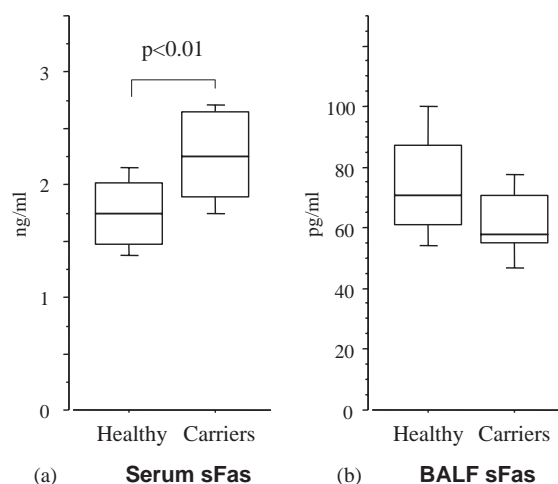
All values were expressed as mean  $\pm$  sd. The Mann-Whitney *U*-test was used to compare differences between unpaired samples. Correlations between parameters were determined by Spearman's rank

correlation coefficient. *P* values less than 0.05 were regarded as significant.

## Results

### Characteristics of BALF cells

The characteristics of BALF cells are summarized in Table 1. Carriers had a significant higher total number of leukocytes in BALF and higher percentages of lymphocytes, CD3+, and CD4+ T-cells, and CD4/CD8 ratio compared with healthy



**Figure 1** Soluble Fas (sFas) concentrations in serum (a), and BALF (b) of healthy subjects and asymptomatic carriers (ACs). The box and whisker plots represent 25–75th percent of results inside the box, the median is indicated by the horizontal bar across the box, and the whiskers on each box represent the 10–90th percentiles. The serum sFas concentrations of ACs are significantly higher than those of healthy subjects.

**Table 1** BALF cell findings in control subjects and asymptomatic HTLV-1 carriers.

	Healthy subjects	Asymptomatic carriers
Total cells $\times 10^5/\mu\text{l}$	$1.55 \pm 1.09$	$2.26 \pm 0.97^*$
Macrophages (%)	$87.4 \pm 6.9$	$77.4 \pm 13.2^\dagger$
Lymphocytes (%)	$9.8 \pm 6.6$	$19.6 \pm 13.4^\dagger$
Neutrophils (%)	$2.2 \pm 3.9$	$2.9 \pm 3.9$
Eosinophils (%)	$0.6 \pm 1.2$	$0.3 \pm 0.6$
CD3+ (%)	$78.5 \pm 11.9$	$87.6 \pm 8.3^*$
CD4+ (%)	$35.0 \pm 12.2$	$55.3 \pm 11.8^*$
CD8+ (%)	$42.3 \pm 13.4$	$35.6 \pm 13.4$
CD4/CD8	$0.99 \pm 0.57$	$2.01 \pm 1.40^*$
CD3+ CD25+ (%)	$5.6 \pm 4.9$	$12.0 \pm 13.2$

Values are expressed as mean  $\pm$  sd.

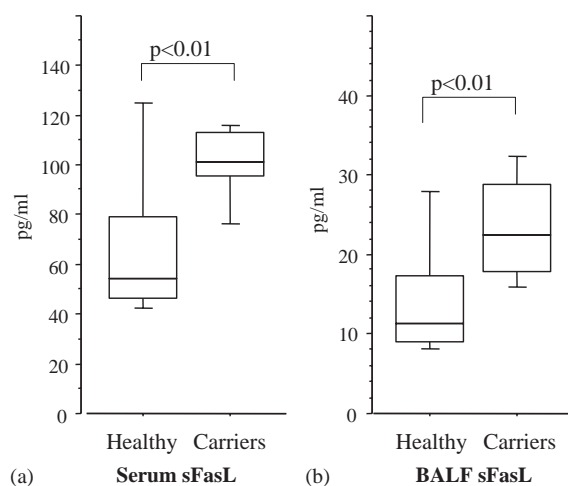
\**P* < 0.01, compared with healthy subjects.

$^\dagger P$  < 0.05, compared with healthy subjects.

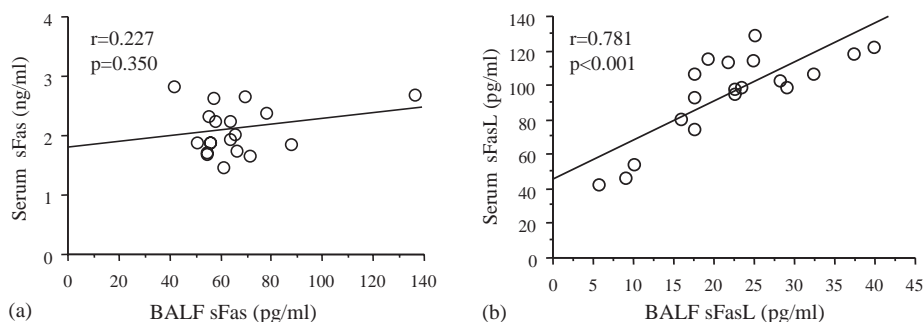
subjects. However, there was no significant difference in the percentage of CD3 + CD25 + T-cells between the two groups.

### sFas and sFasL concentrations in serum and BALF

The mean serum sFas concentration in asymptomatic HTLV-I carriers ( $2.2 \pm 0.4$  ng/ml) was significantly higher than in healthy subjects ( $1.7 \pm 0.3$  ng/ml,  $P = 0.003$ , Fig. 1a). There was no significant difference in BALF concentrations of sFas between the two groups (control:  $73.9 \pm 16.2$  pg/ml, carriers:  $65.1 \pm 22.0$  pg/ml,  $P = 0.06$ , Fig. 1b). Both the serum and BALF concentrations of sFasL were significantly higher in HTLV-I carriers (serum:  $98.9 \pm 21.1$  pg/ml, BALF:  $23.9 \pm 7.1$  pg/ml) than in healthy subjects (serum:  $66.7 \pm 32.6$  pg/ml,  $P = 0.006$ ; BALF:  $14.8 \pm 8.8$  pg/ml,  $P = 0.002$ , Fig. 2a and b).



**Figure 2** sFasL concentrations in serum (a), and BALF (b) of healthy subjects and asymptomatic carriers (ACs). The serum and BALF sFasL concentrations of ACs are significantly higher than those of healthy subjects.



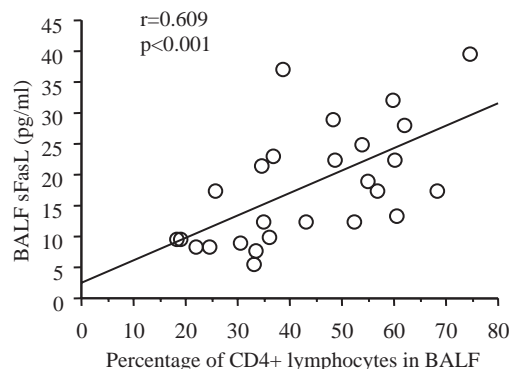
**Figure 3** Correlation between serum soluble Fas (sFas) and BALF sFas (a), and serum sFasL and BALF sFasL concentrations (b) in healthy subjects and asymptomatic carriers. BALF sFasL concentrations correlate significantly with serum sFasL concentrations.

### Correlations between serum and BALF concentrations of sFas or sFasL

There was no correlation between serum and BALF sFas concentrations (Fig. 3a). But serum sFasL concentrations correlated significantly with BALF sFasL concentrations (Fig. 3b) in the control and asymptomatic carriers. In addition, there was significant correlation between serum sFas and serum sFasL concentrations ( $r = 0.554$ ,  $P < 0.001$ , data not shown) and no correlation between BALF sFas and BALF sFasL concentrations ( $r = 0.039$ ,  $P = 0.828$ , data not shown).

### Correlations between BALF sFasL concentrations and BALF cells

BALF sFasL concentrations correlated with the percentage of CD4 + T-cells in BALF of healthy subjects and carriers (Fig. 4). However, there was no significant correlation between BALF sFas concentrations and BALF cells.



**Figure 4** Correlation between BALF sFasL concentrations and the percentage of CD4 + lymphocytes in BALF with healthy subjects and asymptomatic carriers. BALF sFasL concentrations correlate significantly with the percentage of CD4 + lymphocytes.

## Discussion

The major findings of the present study were the presence of high concentrations of sFasL in BALF, and sFas and sFasL concentrations in serum of asymptomatic HTLV-I carriers. Several studies have shown that HAM/TSP is frequently associated with T-lymphocytic alveolitis.<sup>7,8</sup> A similar pulmonary involvement has also been observed in asymptomatic carriers.<sup>9</sup> We previously demonstrated the presence of high percentages of CD3+CD25+ T-cells, i.e., activated T-cells, in BALF of HAM/TSP patients and asymptomatic carriers.<sup>25</sup> In addition, Kawakami and co-workers<sup>26</sup> reported that the histopathological changes in the lung tissues of HTLV-1 transgenic mice showed inflammatory changes with bronchoalveolar lymphocytosis that resembled the histopathological findings observed in HTLV-1-infected patients. We have also reported that HTLV-1 tax mRNA, encoding the transcriptional transactivator Tax, was more frequently detected in BALF cells compared with peripheral blood mononuclear cells and was closely associated with infiltration of activated T-lymphocytes in lung tissue.<sup>27</sup> Furthermore, the expression of tax mRNA was detected in BALF cells of all HAM/TSP patients and even eight of 17 asymptomatic carriers. It is well known that Tax plays a crucial role in the inflammatory response in the lungs of HTLV-1-infected individuals by upregulating the viral promoter and expression of various cellular genes.<sup>28-30</sup> Tax can also induce the expression of the FasL gene in CD4+ T-cell line.<sup>31</sup> Therefore, our present findings demonstrating the presence of high concentrations of sFasL in the serum and BALF of HTLV-1 carriers and a significant correlation between sFasL concentrations and the percentage of CD4 positive T-cells in BALF are not unexpected. It has been shown that human T-cell lines expressing Tax were resistant to apoptosis signals triggered through Fas receptor, and T-cells derived from patients with TSP/HAM and from transgenic mice carrying the env-pX region of HTLV-1 increased resistance to Fas apoptosis.<sup>32</sup> It has also been reported that the inactivated supernatants of some HTLV-1-positive T-cell lines can transfer resistance to apoptosis to uninfected cells.<sup>33</sup> These data suggests that HTLV-1 infected T-cells are resistant to apoptosis and increased in lung tissue, and thus secretion, such as sFasL, from HTLV-1 infected cells inhibits apoptosis to uninfected T-lymphocytes. Together with our present results, T-lymphocytic alveolitis by down-regulating Fas-FasL mediated apoptosis may be observed in lungs of asymptomatic carriers, although the additional clinical and in vitro studies will need to refute our

hypothesis. Human immunodeficiency virus (HIV), which is the same kind of human retroviral infection, also induces the expression of FasL. By upregulating FasL, the infected cells evade apoptosis induced by cytotoxic T-lymphocytes.<sup>34</sup> However, asymptomatic infected individuals do not show the lymphocytic alveolitis in the lung,<sup>35</sup> so it would be interesting to clear what are the differences in T-cell alveolitis and in the role of the Fas/FasL system between HTLV-1 and HIV infections.

In the present study, the serum concentrations of sFas in carriers were also significantly higher than in healthy subjects. Previous studies have shown significantly higher mFas positive rate on T-cells and serum sFas concentrations in HAM/TSP and ATL patients compared with healthy subjects. Kamihiro et al.<sup>23</sup> reported a positive correlation between sFas and soluble interleukin-2 receptor (sIL-2R) in serum of HTLV-1 infected patients. They also demonstrated that serum sFas concentrations significantly correlated with various clinical parameters, such as serum lactic dehydrogenase activity, tumor burden, hypercalcemia and prognosis in ATL,<sup>36</sup> and suggested that sFas and sIL-2R are of the same origin, and were possibly derived from activated-T-cells or leukemic-T-cells. In the present study, the percentage of CD3+CD25+ (IL-2R), a marker of activated-T-cells, in peripheral blood of carriers was higher than in healthy subjects (data not shown). These results suggest that serum sFas derived from activated-T-cells and/or HTLV-1 infected cells, may block apoptotic cell death by inhibiting the binding of mFasL to mFas and that HTLV-1 infected cells may be persistently present in HTLV-I infected patients. Tax also activates a number of cellular genes, including IL-2 and IL-2R $\alpha$ .<sup>37</sup> IL-2 is known to upregulate anti-apoptotic members of the BCL-2 family. IL-2 also promotes Fas-dependent activation-induced cell death by upregulating Fas and FasL.<sup>38</sup> Thus, the role of IL-2 in apoptosis is now controversial, but it is possible that these other cytokines also regulate the lymphocyte apoptosis in the lung of HTLV-1 carriers.

We found no significant difference in BALF sFas concentrations between carriers and healthy subjects, and no correlations between BALF sFas concentrations and BALF cells. Kuwano et al.<sup>39,40</sup> demonstrated that the expression of mFas was upregulated in bronchoalveolar and alveolar epithelial cells in lung tissues from patients with idiopathic pulmonary fibrosis (IPF) but sFas concentrations in BALF were not significantly higher in IPF patients compared with control subjects and did not correlate with the number of inflammatory cells. These results indicate that the production of



sFas concentrations in BALF may be derived from not only lymphocytes but also other cells.

In our study, the mean age of the healthy controls was considerably lower compared with the HTLV-1 carriers. Gupta have observed increased expressions of Fas and FasL in T-cells from aging subjects as compared with healthy young control.<sup>41</sup> Seishima and colleagues have also reported that the levels of sFas in serum were increased with aging.<sup>42</sup> Therefore, it could be the reason why the serum levels of sFas in the HTLV-1 carriers were higher than those in the healthy controls in this study. However, the serum levels of sFasL have been reported to be decreased with aging,<sup>43</sup> so our findings demonstrating the higher levels of sFasL in HTLV-1 carriers did not merely reflect the influence of age.

In conclusion, we have demonstrated in the present study the presence of elevated concentrations of sFas and sFasL in serum and sFasL in BALF of asymptomatic HTLV-I carriers relative to those of healthy subjects. We also found a significant correlation between the concentrations of sFasL and percentage of CD4<sup>+</sup> T-cells in BALF. Although we have not directly demonstrated the correlation between sFasL levels in BALF and the inhibition of lymphocyte apoptosis, our findings speculate that overproduction of sFasL in the lung of asymptomatic HTLV-1 carriers perpetuates the inflammatory condition and leads to T-lymphocytic alveolitis by down-regulating Fas-mediated apoptosis.

## Acknowledgements

The authors thank Atsushi Yokoyama for his technical assistance. This study was supported in part by a research grant from the Ministry of Education, Science, Sports, and Culture of Japan.

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